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Heterologous expression of nonribosomal peptide synthetases in *B. subtilis*: construction of a bi-functional *B. subtilis*/*E. coli* shuttle vector system

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Abstract

A major obstacle in investigating the biosynthesis of pharmacologically important peptide antibiotics is the heterologous expression of the giant biosynthetic genes. Recently, the genetically engineered strain *Bacillus subtilis* KE30 has been reported as an excellent surrogate host for the heterologous expression of an entire nonribosomal peptide synthetase (NRPS) gene cluster. In this study, we expand the applicability of this strain, by the development of four *Escherichia coli*/*B. subtilis* shuttle expression vectors. Comparative overproduction of hybrid NRPS proteins derived from both organisms revealed a significant beneficial effect of overproducing proteins in *B. subtilis* KE30 as underlined by the production of stable nondegradative proteins, as well as the formation of active phosphopantetheinylated holo-proteins.

Keywords: Nonribosomal peptide synthetase; Heterologous expression; T5 phage promoter; *Bacillus subtilis*

1. Introduction

Nonribosomal peptide synthetases (NRPSs) are complex multimodular enzymes that catalyze the biosynthesis of many pharmacologically important peptides. Significant information about these giant mega-enzyme complexes could be obtained from investigation of NRPSs derived from genus *Bacillus* [1–4]. Several gene fragments originating from this organism could be successfully expressed in the heterologous host *Escherichia coli*, and the encoded NRPS domains and modules could be biochemical characterized [5–7]. However, although many techniques are available for *E. coli*, several limitations for heterologous expression of NRPS genes have been encountered.

In *E. coli*, the major challenge regarding overproduction of multimodular NRPSs is the ability to synthesize these giant proteins in a properly folded way [8,9]. Other enzymes (i.e. 4′-phosphopantetheinyl transferases (PPTases) and type II thioesterases), whose genes are associated with NRPS biosynthetic gene cluster, also play an important role in nonribosomal peptide (NRP) formation such as posttranslational modification and editing of misprimed peptidyl carrier protein (PCP) domains. Phosphopantetheinylation of PCP domains is catalyzed by specialized PPTase, which is usually not present in commonly used *E. coli* host strains [10,11]. *Bacillus subtilis*, a natural producer of NRPs, harbors a corresponding PPTase gene, whose gene product, Sfp, is responsible for the apo-to-holo conversion of PCP domains [2]. However, a drawback of gene expression here is the instability of recombinant plasmids in *B. subtilis*. This leaves chromosomal integration the only suitable way to stably maintain cloned genes in this organism [12,13].

In the presented study, a shuttle vector system was developed that allows parallel gene expression in *E. coli* and

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Abbreviations: NRPS(s), nonribosomal peptide synthetase(s); PCP, peptidyl carrier protein; PPTase, 4′-phosphopantetheinyl transferase; IPTG, isopropyl-β-D-thiogalactoside

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B. subtilis. While the plasmids are self-replicating in *E. coli*, a stable copy of the cloned gene is maintained by chromosomal integration in *B. subtilis* KE30, a recently reported surrogate host for heterologous overexpression of an entire NRPS gene cluster [14]. The tightly regulated, isopropyl-β-D-thiogalactoside (IPTG)-inducible T5 P_{N25} phage promoter controls gene expression in both organisms [15,16]. In order to demonstrate the superiority over *E. coli*, we show the production of nondegradative, posttranslationally modified NRPS holo-proteins in *B. subtilis*. We believe that in combination with a developed *E. coli* cloning system, the constructed shuttle vectors may facilitate access to the production and manipulation of so far unattainable NRPS systems.

2. Materials and methods

2.1. Strains and growth conditions

E. coli was grown in rich medium 2×YT or [17]. *B. subtilis* was grown in 2×YT or Difco Sporulation medium [18]. For *Bacillus* cultures erythromycin, lincomycin, chloramphenicol and kanamycin were used at final concentrations of 25, 1, 5, 10 μg ml^{-1}, respectively. In *E. coli*, kanamycin and ampicillin were used at final concentrations of 25 and 100 μg ml^{-1}, respectively.

2.2. PCR amplification and plasmid construction

DNA was amplified from chromosomal DNA of the surfactin producer *B. subtilis* ATCC 21332. PCR amplification and DNA manipulations were performed as described previously [14].

Plasmids pSD193, pSD270, pKE151 and pKE170 are derivatives of pKE18 [14]. A 146-bp fragment was amplified from plasmid pQE60 (Qiagen, Hilden, Germany) using the oligonucleotides (restriction sites for subsequent cloning in bold, modified sequences in underlined, further restriction sites in italic) 5′-AAT **ACA** **TGT** CCT TTC GTC TTC ACC TC-3′ (5′T5(*Nsp*I)) and 5′-T**GG** **ACC** **CAT** **GG**T AAT TTC TCC TCT-3′ (3′T5(*Nco*I)), modified with *Nsp*I and *Nco*I and ligated into pKE18 (*Sph*I/*Nco*I), to give pSD181. A 110-bp DNA fragment was amplified from plasmid pQE70 using the oligonucleotides 5′-A**AA** **CCA** **TGG** *GCA TCC* GAG *GAT CCT* G-3′ (5′pQE70-MCS(*Nco*I)) and 5′-T**AT** **AGA** **TCT** CTG AGG TCA TTA CTG G-3′ (3′pQE70-down*His*$_6$(*Bgl*II)) modified with *Nco*I and *Bgl*II and ligated into pSD181 (*Nco*I/*Bam*HI), to give pSD185. The 1494-bp *kan* resistance cassette was amplified from pDG783 [19] using the oligonucleotides 5′-A**AT** **CTG** **CAG** AGC GAA CCA TTT GAG GTG A-3′ (5′*kan*(*Pst*I)) and 5′-**ATA** **CTG** **CAG** ATA CAA ATT CCT CGT AGG C-3′ (3′*kan*(*Pst*I)) modified with *Pst*I and ligated into pSD185 (*Pst*I), to give pSD193.

For construction of pSD270, pSD185 was re-amplified using inverse PCR with the oligonucleotides 5′-GG**C** **GTT** **AAC** *GGA TCC* GGA TCT CA-3′ (5′psrfT5/*His*$_6$(*Hpa*I)) and 5′-**AAG** **GTT** **AAC** TC*G CAT GCC CAT G*-3′ (3′psrfT5/*His*$_6$(*Hpa*I)). The 6707-bp DNA fragment was terminally modified with *Hpa*I and *Dpn*I and religated to give pSD237. pSD237 was treated with *Pst*I and the *kan* resistance cassette was inserted in an analogous way to pSD193. The resulting plasmid was designated pSD270. For the construction of pKE151 and pKE170, alternative multiple cloning sites (MCSs) were integrated using short DNA fragments containing 5′ and 3′ sticky-ends of the corresponding restriction sites. For this purpose, the designed oligonucleotides were phosphorylated using polynucleotide kinase, denaturated at 95°C and subsequently annealed at 4°C. After phosphorylation and annealing of the oligonucleotides 5′-**AAT** **TCA** TTA AAG AGG AGA AAT TA*C ATA TGG GCA TGC TTA ATT AAG*-3′ (5′MCS(*Nde*I)) and 5′-**GAT** **CC***T TAA TTA AGC ATG CCC ATA TGT* AAT TTC TCC TCT TTA ATG-3′ (3′MCS(*Nde*I)), the DNA fragment was ligated in pSD270 (*Eco*RI/*Bam*HI) to give pKE151. For the construction of pKE170, the phosphorylated and annealed oligonucleotides 5′-**TAT** **GG***G CAT GCG GCT AGC* GG-3′ (5′MCS(*Nhe*I)) and 5′-**GAT** **CCC** *GCT AGC CGC ATG CCC* **A**-3′ (3′MCS(*Nhe*I)) were ligated in pKE151 (*Nde*I/*Bam*HI). An internal 4927-bp DNA fragment, part of *srfA-B*, was amplified using the oligonucleotides 5′-TAA **GCA** **TGC** TTT CAT *CTG CAG* AAA C-3′ (5′AspLeu *srfB*(*Sph*I)) and 5′-**AAT** **GGA** **TCC** TTC GGC ACG CTC TAC-3′ (3′AspLeu *srfB*(*Bam*HI)) and modified with *Sph*I and *Bam*HI. pQE70 (Qiagen, Hilden, Germany) was digested in the same manner and both fragments were ligated to give pSD142.

A 1332-bp DNA fragment, part of *srfA-A*, was amplified using the oligonucleotides 5′-TAT **GCA** **TGC** AAA TAA CTT TTT ACC CTT-3′ (5′*srfA1-C*(*Sph*I)) and 5′-ATA **CTG** **CAG** CAG AAA TCA GTG TTA ATT CA-3′ (3′*srfA1-C*(*Pst*I)) modified with *Sph*I and *Pst*I and ligated into pSD142 (*Sph*I/*Pst*I) to give pSD164. The 6243-bp gene was cut out from pSD164 using *Sph*I and *Bam*HI. The fragment was ligated into pSD193 and pKE170 (*Sph*I/*Bam*HI) to give pSD197 and pKE203, respectively.

2.3. Transformation of B. subtilis

Transformation of *B. subtilis* was performed as described previously [14].

2.4. Expression in E. coli

Expression of the recombinant NRPS system in *E. coli* was carried out after transformation of pKE203 in M15[pREP4]. Expression was carried out as described previously [3,20]. Cells were broken by two passages through a French Press cell (Amicon).

2.5. Expression in Bacillus

Pre-warmed 2×YT medium (400 ml) was inoculated 1/100 with an overnight culture of the corresponding *Bacillus* strain and allowed to grow at 30°C under rigorous shaking to an optical density of A_{600} 0.7. Cells were induced with 0.1 mM IPTG and were grown for an additional 2 h before being harvested. Cells were pelleted by centrifugation, resuspended in 5 ml of SMM^+ buffer (20 mM sodium malonate, 20 mM $MgSO_4$, 500 mM sucrose, 0.5 mg/ml lysozyme, pH 6.5) and subsequently incubated at 37°C for 45 min. Protoplasts were broken by three passages through a French Press cell (Amicon).

2.6. Purification of recombinant NRPSs using IMAC

The purification of His_6-tagged proteins was performed using Ni^{2+}-affinity chromatography [6]. Expression and purity were determined by SDS–PAGE using Laemmli gel systems [21]. Concentrations of proteins were determined by Bradford test [22].

2.7. Purification of a recombinant dimodular NRPS from the strain SD10

All following steps were carried out at 4°C. The supernatant, obtained after centrifugation of lysed *B. subtilis* cells, was subjected to a 55% ammonium sulfate precipitation. The precipitate was pelleted by centrifugation, the pellet was resuspended in 2 ml of buffer A (50 mM HEPES, 100 mM sodium chloride, pH 6.5) and dialyzed against 100 volumes of the same buffer for 4 h. The solution was applied to a HiLoad™ 26/60 Superdex 200 column (Amersham Pharmacia Biotech, Freiburg, Germany) previously equilibrated to buffer A. Elution of the proteins was performed isocratic at a constant flow rate of 5 ml min^{-1}. Fractions containing the recombinant protein were identified by SDS–PAGE [21], and pooled (as repeated after each purification step). The obtained sample was applied to a HiLoad™ 26/10 Q-Sepharose column (Amersham Pharmacia Biotech, Freiburg, Germany) previously equilibrated to 10% buffer B (50 mM HEPES, 1 M sodium chloride, pH 6.5). The flow rate was 0.5 ml min^{-1}, and elution of proteins was accomplished by applying a linear gradient to 100% buffer B from 10 to 50 min. Subsequently, the protein pool was dialyzed against 100 volumes buffer C (50 mM HEPES, 1 M ammonium sulfate, pH 7.0) for 4 h. Following centrifugation, the sample was applied to a HiLoad™ 26/10 Phenyl Sepharose column (Amersham Pharmacia Biotech, Freiburg, Germany) previously equilibrated to buffer C. The flow rate was 2 ml min^{-1}, and elution of proteins was accomplished by applying a linear gradient to buffer D (50 mM HEPES, pH 7.0).

2.8. ATP–pyrophosphate exchange assay

In order to test the adenylation activity of the recombinant hybrid NRPSs, amino acid-dependent ATP–pyrophosphate exchange assays were performed as described previously [6].

2.9. Assay for detection of covalent amino acid incorporation

In order to measure covalently bound amino acids, and to access the degree of apo-to-holo conversion of the recombinant hybrid NRPSs, a thioester formation assay was carried out as described previously [6].

Using the recombinant dimodular NRPS overproduced in *B. subtilis* SD10, dATP instead of ATP was used. Furthermore, on account of the lower protein concentration only 200 nM of enzyme could be used.

2.10. In vitro modification of the PCP domain

To convert apo-forms of PCP domains to their active holo-form, proteins were phosphopantetheinylated in vitro using the recombinant PPTase Sfp as described elsewhere [10].

3. Results and discussion

3.1. Construction of a set of E. coli/B. subtilis shuttle vectors

In the presented study, we describe the construction and utilization of a set of four newly developed *E. coli/B. subtilis* shuttle vectors, designated pSD193, pSD270, pKE151 and pKE170 (Fig. 1). The following five prerequisites were imposed on the construction of the shuttle vectors: (1) in *B. subtilis*, maintenance of a stable copy of the cloned gene should be achieved by chromosomal integration; (2) to benefit from sophisticated *E. coli* cloning techniques, the vectors should contain an *E. coli* origin of replication; (3) an unique promoter, functional in *E. coli* and *B. subtilis*, should allow the parallel expression of genes in both organisms; (4) expression should be tightly regulated until induction; and (5) in optimized distance to a ribosomal binding site (RBS), an ATG start codon should allow expression of gene fragments that lack a native start codon.

In order to meet those demands the following genes and features were introduced into the different shuttle vectors:

1. All vectors contain the genes *yckGH* (2.5 kb) and *ycxA'* (0.9 kb), which are used to facilitate a stable integration in the *srfA*-deletion strain *B. subtilis* KE30 [14]. Double crossover integration takes place into the former *srf*A chromosomal locus and can be monitored by selectable markers (see Fig. 2).

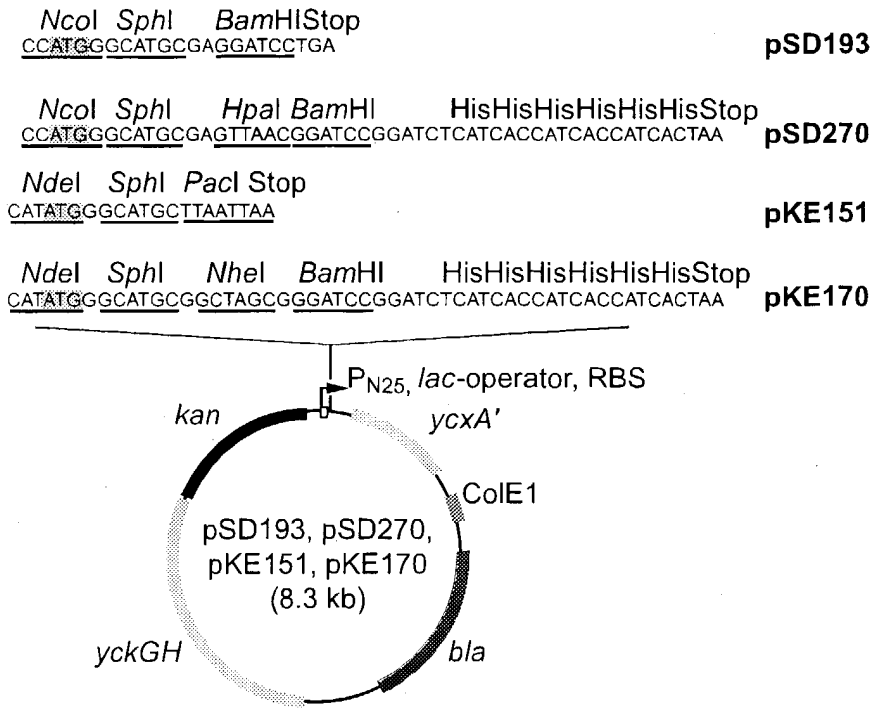

Fig. 1. Physical map of the *E. coli*/*B. subtilis* shuttle vectors pSD193, pSD270, pKE151 and pKE170. Shown are the 5′ and 3′ homologous regions for integration to the former *srfA* locus in *B. subtilis* KE30 (*yckGH* and *ycxA'*), the *E. coli* ColE1 origin of replication, the resistance markers *bla* and *kan*, conferring ampicillin and kanamycin resistance, respectively. Indicated as an arrow is the site of T5 P_{N25} phage promoter, the *lac* operator, as well as the location of the RBSII location. The relevant nucleotide sequences of the multiple cloning sites, His_6-tag coding region and stop codon in pSD193, pSD270, pKE151 and pKE170 are shown in detail.

2. In *E. coli*, the plasmids are self-replicating and can be selected for ampicillin and kanamycin resistance.
3. In the constructed vector systems, expression of cloned genes is maintained under the control of the T5 P_{N25} phage promoter. In contrast to the phage promoter T7, which is recognized by the inherent phage RNA polymerase, the T5 promoter is also accepted by the vegetative *E. coli* RNA polymerase [15]. Beyond, the T5

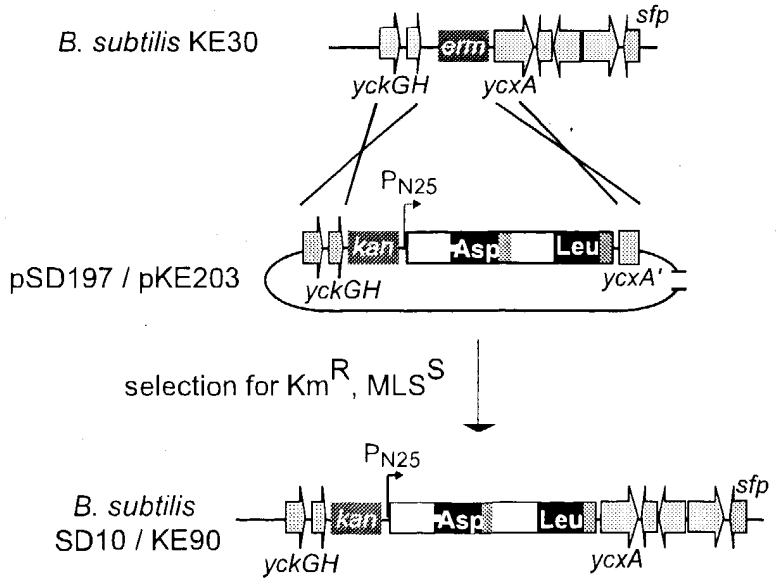

Fig. 2. Insertion of a dimodular hybrid peptide synthetase gene in *B. subtilis* KE30. Schematic diagram showing the construction of the *B. subtilis* strains SD10 and KE90. Top, chromosomal locus of the *srfA*-deletion site in *B. subtilis* KE30 [14]. Transformation of *B. subtilis* KE30 with the integration plasmids pSD197 and pKE203 resulted in the chromosomal integration of the dimodular hybrid NRPS gene $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$, yielding the *B. subtilis* strains SD10 and KE90, respectively (bottom).

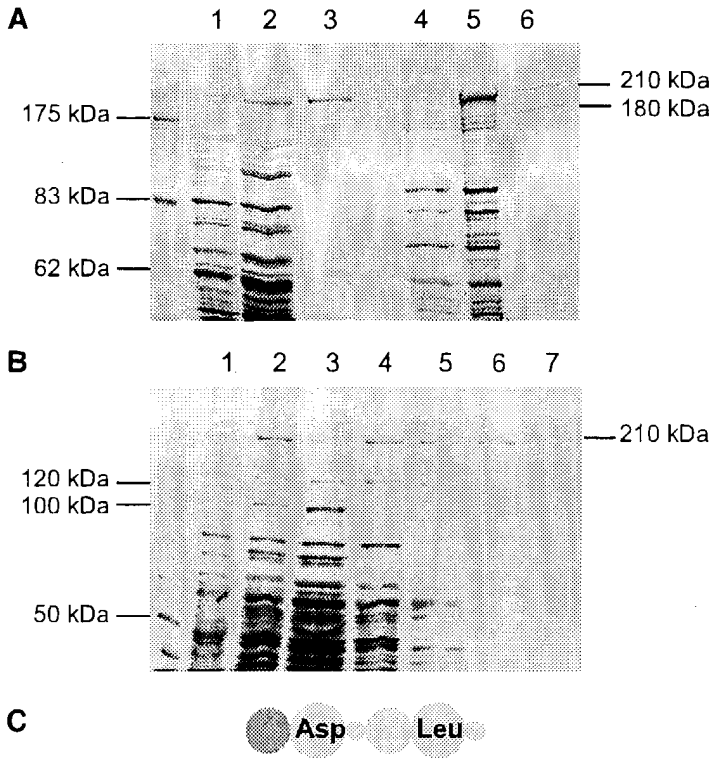

Fig. 3. Expression of recombinant dimodular NRPS genes in *E. coli* and *B. subtilis*. A: Coomassie blue-stained SDS–PAGE confirming expression of $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$-His_6 in *B. subtilis* KE90 (lanes 1–3), and *E. coli* M15 [pREP4,pKE203] (lane 4–6), as well as subsequent purification using Ni^{2+}-NTA affinity chromatography. Lanes 1 and 4: Total cellular protein before induction; lanes 2 and 5: total cellular protein after 2 h induction with 0.1 mM IPTG; and lanes 3 and 6: dialyzed protein pools after Ni^{2+}-NTA affinity chromatography purification. Utilizing heterologous expression in *E. coli* M15 [pREP4,pKE203], a partial degradation of the recombinant 210-kDa protein occurs, as detectable by the appearance of a second inducible protein band at about 180 kDa (lanes 4–6). B: Coomassie blue-stained SDS–PAGE confirming expression of $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$ in *B. subtilis* SD10. Total cellular protein before (lane 1) and 2 h after induction with 0.1 mM IPTG (lane 2). Lane 3: Supernatant of broken cells after French Press. Lane 4: Resuspended proteins after 55% ammonium sulfate precipitation. Lane 5: Protein pool after size exclusion chromatography (Sephadex 200). Lane 6: Protein pool after anion exchange chromatography (Q-Sepharose). Lane 7: Protein pool after hydrophobic interaction chromatography (Phenyl-Sepharose). C: Schematic presentation of the dimodular hybrid NRPS constructed. The domains used for engineering are highlighted.

promoter sequence resembles the vegetative sigma factor-dependent promoter sequence in *B. subtilis* [16]. *B. subtilis* expression vectors, exploiting the T5 promoter, have been described previously [23].

4. The plasmids provide an optimized promoter–operator element consisting of the T5 promoter and two modified *lac*-operator sequences. A chromosomal copy of *lacI* is available in the *amyE*-locus of *B. subtilis* KE30 [14].
5. The MCSs introduced in pSD193, pSD270, pKE151 and pKE170 permit translation of internal gene fragments (Fig. 1). An ATG start codon is embedded into the recognition sites of the restriction endonucleases *Nco*I (C**CATGG**) and *Nde*I (**CATATG**), respectively, and has been positioned in optimized distance to the T5 promoter and the synthetic RBSII. In plasmids pSD270 and pKE170, a C-terminal His_6-tag can be fused to the translated proteins.

3.2. Overproduction of a recombinant dimodular peptide synthetase in E. coli and B. subtilis KE30

All expression vectors constructed were tested for their performance in *E. coli* M15[pREP4] and *B. subtilis* KE30. Exemplary we describe here the comparative expression of a dimodular hybrid NRPS gene, which encodes the hybrid synthetase $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$ (Fig. 3C). This construct artificially links two NRPS domains, with an internal fragment of the surfactin synthetases B (A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$ N-terminally fused to the condensation domain of SrfA-A1 [2]. The resulting hybrid gene was cloned into the *E. coli*/*B. subtilis* shuttle vectors to compare heterologous production of the hybrid NRPS in both host organisms. Cloning into pSD193 and pKE170 (Fig. 1) yielded the expression plasmids pSD197 and pKE203, respectively.

First, production of the hybrid dimodular NRPS in its

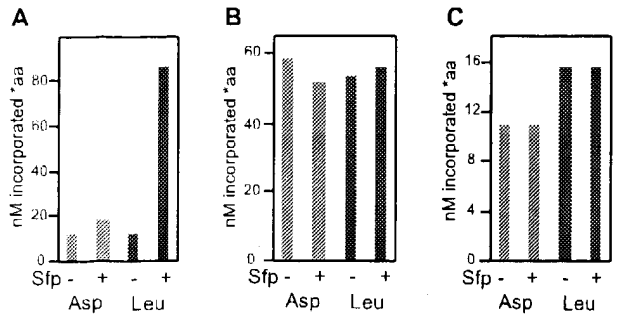

Fig. 4. Covalent amino acid incorporation into the dimodular hybrid NRPSs. The comparative thiolation assay was performed using the recombinant protein $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$-His_6 derived from *E. coli* M15[pREP4,pKE203] (A) and *B. subtilis* KE90 (B), as well as the untagged derivative $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$ obtained from *B. subtilis* SD10. The purified proteins were used straight as obtained after purification and after supplementary modification using the PPTase Sfp. The assays were performed using the radiolabeled substrate amino acids L-[^{14}C]aspartate, and L-[^{14}C]-leucine. In a control, no ATP was used (not shown). The reduced level of amino acid incorporation observed for the recombinant protein derived from *B. subtilis* SD10 can be related to lower protein concentration used for the thioester-formation assay.

His_6-tagged form was investigated. The expression plasmid pKE203 constructed was transformed in *E. coli* M15[pREP4] and *B. subtilis* KE30, respectively. In the latter case, correct and stable chromosomal integration was achieved by selecting for MLS-sensitive and kanamycin-resistant strains, and could be confirmed by 5′- and 3′-terminal sequencing for six transformants. The obtained *B. subtilis* overproducer of $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$-His_6 was designated KE90 (Fig. 2). Next, expression of the hybrid NRPS gene construct was analyzed in *E. coli* and *B. subtilis*, and overproduction of an IPTG-inducible protein of the expected size (about 210 kDa) could be determined in both organisms (Fig. 3A). However, single-step Ni^{2+}-NTA affinity chromatography revealed a partial degradation of the *E. coli*-derived protein, leading to an additional protein of about 180 kDa (Fig. 3A). The putative degradation product was already present in total cellular preparations of *E. coli*. Strikingly, the *B. subtilis*-derived hybrid protein $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$-His_6 apparently remained stable.

In order to verify this observation and to show that overproduction of NRPSs in *B. subtilis* is in fact advantageous, the previous experiment was repeated for the untagged variant of the NRPS hybrid protein. The corresponding gene fragment $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$ was cloned into the *E. coli*/*B. subtilis* shuttle vector pSD193, yielding the expression plasmid pSD197. Again, the expression plasmid constructed was transformed in *E. coli* M15[pREP4] and *B. subtilis* KE30, and stable chromosomal integration in *B. subtilis* was achieved by selecting for MLS-sensitive and kanamycin-resistant strains. Correct integration could be confirmed by 5′- and 3′-terminal sequencing for 12 transformants, and the obtained *B. subtilis* overproducer of $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$ was designated SD10 (Fig. 2). Subsequent overproduction of the hybrid NRPS again revealed an intrinsic instability of the *E. coli*-derived protein (data not shown). In contrast, no degradation could be observed in *B. subtilis*. This latter protein was partially purified using 55% ammonium sulfate precipitation, size exclusion, anion exchange and hydrophobic interaction chromatography (Fig. 3).

3.3. Biochemical characterization of the recombinant dimodular peptide synthetases

As deduced from the domain structure of the recombinant dimodular NRPS $C_{srfA-A1}$-(A_{Asp}-PCP-C-A_{Leu}-PCP)$_{srfA-B2-3}$, the hybrid protein should be capable to activate the substrate amino acids L-Asp (SrfA-B2) and L-Leu (SrfA-B3) as amino acyl adenylates, and to incorporate them in covalent thioester binding. Thus, substrate selectivity of the hybrid NRPS was investigated by applying amino acid-dependent ATP–pyrophosphate exchange reactions. For the recombinant protein heterologously produced in *E. coli* M15[pREP4,pKE203], a specific activation of L-Asp and L-Leu could be measured, while no activation of the miscognate substrate L-Phe was observed. Likewise, a comparable selectivity could be observed for the dimodular hybrid NRPSs derived from *B. subtilis* SD10 and KE90, although activation efficiency for L-Asp was slightly reduced.

Subsequently, the covalent incorporation of radiolabeled amino acids was investigated by using the thioester-formation assay (Fig. 4). Here, the extent of post-translational modification was analyzed by a comparative in vitro modification with the PPTase Sfp. In the case of the dimodular NRPS protein heterologously produced in *E. coli* M15[pREP4,pKE203], virtually no incorporation of radiolabeled amino acids L-[^{14}C]Asp and L-[^{14}C]Leu could be detected. This indicates that the PCP domains were for the most part not converted in vivo from inactive apo to active, phosphopantetheinylated holo-form in the heterologous host *E. coli*. Following in vitro modification with the PPTase Sfp, however, an increased level of radiolabeled amino acid incorporation could be observed. In contrast, for hybrid NRPS proteins derived from *B. subtilis* SD10 and KE90, the capability to form aminoacyl-*S*-(co-factor 4′-phosphopantetheine) thioesters was shown to be independent of a preceding in vitro modification using Sfp, and here, no further increase in thioester formation could be obtained. Apparently, due to the presence of the native PPTase Sfp in *B. subtilis* [2], the NRPSs are produced in their active, pantetheinylated holo-form.

4. Conclusion

In the presented study, we report on a set of four de-

veloped shuttle vectors that allow parallel expression of recombinant genes in the heterologous hosts *B. subtilis* and *E. coli*. By the example of a T5 promoter controlled expression of a dimodular hybrid NRPS, the advantageous utilization of *B. subtilis* for heterologous NRPSs production could be demonstrated. In contrast to *E. coli*, the proteins could be obtained in a stable, nondegradative and posttranslationally modified active holo-form.

Due to the different codon usage in Gram-positive bacteria, heterologous expression in *B. subtilis* may be advantageous compared to *E. coli* under certain conditions. Thus, notwithstanding the fact that this report focuses on heterologous expression of NRPS (hybrid) genes, we want to emphasize that the developed vectors also provide a useful tool for any *B. subtilis*-based gene expression [24]: (1) stable integration can be easily monitored; (2) expression is strong, and growth phase-independent; (3) expression levels can be controlled by adjusting the ratio between IPTG inductor and *Lac*I repressor; and (4) optionally, overproduced proteins can be produced as C-terminal His_6-tag fusions.

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